

Carbonyls in Oxidizing Fat. VI. The Girard T Reagent in the Isolation and Determination of Micro Amounts of N-Aliphatic Aldehydes and 2-Alkanones

SUMMARY

Complete reaction of the Girard T reagent with n-alkanals, alk-2-enals, alk-2,4-dienals, and 2-alkanones of carbon chain length up to C-13 was obtained in anhydrous tertiary butyl alcohol. However, in practical applications in the presence of fat these reactions were greatly retarded and inhibited. Modification by the use of water in the reaction gave quantitative recovery of aldehyde homologous series from the fat. Quantitative or even uniform recovery of 2-alkanones could not be obtained. Optimum reaction with the Girard T reagent was at room temperature, and refluxing tended to decrease yields. Girard T hydrazones were converted quantitatively to 2,4-dinitrophenylhydrazones. The method is useful for the determination of aldehydes generated in autoxidized fats.

The Girard T reagent as a means of isolating carbonyl compounds was first reported by Girard and Sandulesco (1936). Valuable because of the water solubility of its hydrazones, this reagent has been used quite extensively for the isolation of carbonyl compounds from natural products (Wheeler, 1962). There have been a good many misconceptions regarding the Girard T reaction. Because of the reported relative ease of hydrolysis it was considered applicable only for ketones (Girard and Sandulesco, 1936) and therefore specific for that class of compounds. However, it has been shown to be effective for aldehydes, the derivatives being readily decomposed by mineral acid (Forss and Dunstone, 1954).

There is little in the literature on the completeness of reaction and subsequent recovery of aliphatic aldehydes and ketones. The mild conditions possible for the Girard T reagent are attractive for studies of the chemistry of oxidative fat rancidity, and of the related isolation and determination of free carbonyl compounds. Earlier work by the authors (Gaddis *et al.*, 1960) showed the possibilities of such use, but results did not check out in some instances, and a

thorough study of the reagent's application was indicated. Nothing was known concerning the completeness of reaction with the various classes of carbonyl compounds involved, or of the efficiency of conversion of Girard T hydrazones to 2,4-dinitrophenylhydrazones. The latter derivatives have been shown to be useful for the characterization and measurement of n-aldehydes and 2-alkanones by already developed procedures (Ellis *et al.*, 1958; Ellis and Gaddis, 1959; Gaddis *et al.*, 1960; Gaddis and Ellis, 1959a,b).

Preliminary work has indicated that a major error may result from choice of reaction solvent in the Girard T reaction. Methanol and ethanol have been the standard solvents used since it was first reported. Braude and Forbes (1951) reported that 2,4-dinitrophenylhydrazine dehydrogenates primary and secondary alcohols containing two or more ethylenic or phenyl substituents in conjugation with the alcohol group, but observed no influence on saturated alcohols. Gaddis *et al.* (1961) found that both Girard T reagent and 2,4-dinitrophenylhydrazine tend to dehydrogenate or oxidize primary and secondary saturated alcohols in

micro amounts to their corresponding aldehydes or ketones. Tertiary butyl alcohol was found to be a relatively inert solvent that gave negligible blanks. This alcohol is also an excellent solvent for fats. Studies are described in this paper on the completeness of reaction of the Girard T reagent with n-alkanals, alk-2-enals, alk-2,4-dienals, and 2-alkanones under various conditions, and the capability of the reagent in the recovery of these types of compounds from fat.

EXPERIMENTAL

Solvents and reagents. Carbon tetrachloride, benzene, tertiary butyl alcohol, ethanol, methanol, and isopropanol were rendered carbonyl-free by treatment with 3 g trichloroacetic acid and 4-5 g 2,4-dinitrophenylhydrazine per liter, refluxing one hour, and distillation. Hexane was purified by the rapid method of Hornstein and Crowe (1962). The Girard T reagent was recrystallized twice from anhydrous purified ethanol, washed with tertiary butyl alcohol, and stored in a desiccator over concentrated sulfuric acid. The 2,4-dinitrophenylhydrazine reagent in 2*N* HCl was made up fresh and purified as described by Gaddis *et al.* (1959). Authentic carbonyl compounds were distilled twice just before use, and their purity was checked by direct reaction with 2,4-dinitrophenylhydrazine reagent and paper chromatographic examination of the reaction mixture products (Gaddis and Ellis, 1959a). Alkanals and 2-alkanones were obtained from commercial sources. Alk-2-enals were prepared by the method of Radlove (1959), and alk-2,4-dienals by the procedure of Pippen and Nonaka (1958).

Methods. General procedure. The method used was to react 0.5-1.0 g of Girard T reagent in 20.0 ml of tertiary butyl alcohol (or other alcohol) containing an aliquot of a stock solution of the carbonyl compound (0.05-3 mg) in the alcohol.

To determine recovery from fat, 10.0 g of mildly rendered fresh lard were combined with the reaction mixture. To perform the reaction under aqueous conditions, 8.0 ml of water or buffer solution were added to the reaction mixture. For acid catalysis, 2.0 ml of glacial acetic acid (Girard and Sandulesco, 1936), 0.2 g Dowex 50 (Gaddis *et al.*, 1960), or Amberlite IRC 50 (Teitelbaum, 1958) was included. (Trade names are mentioned for identification, implying no endorsement.)

The reaction was conducted under an atmosphere of nitrogen by shaking at room temperature for a minimum of 3 hr. At the end of the reaction period, 150 ml of ice water (containing 0.9 equiva-

lent of NaOH to acetic acid used) were added. After stirring, the contents of the reaction flask were transferred to a blender and agitated 1 min. The mixture was then transferred to a separatory funnel. The reaction flask and blender were washed with 50 ml of ice water, and this was added to the main portion. The contents of the separatory funnel were extracted three times with 35 ml of purified hexane. Reaction flask and blender were washed with 50 ml of hexane, which was added to the hexane extract. The hexane extract was washed with 50 ml of water.

The two aqueous portions were combined and added to 100 ml of 2,4-dinitrophenylhydrazine reagent. One hundred and twenty milliliters of 2*N* HCl and 50 ml of carbon tetrachloride (optional in the case of aldehydes) were added, and the mixture was shaken well for 3 hr at room temperature.

Direct reaction of known amounts of monocarbonyl compounds with 2,4-dinitrophenylhydrazine was performed in two ways: 1) a 1.00-ml portion of a stock solution of the carbonyl compound in tertiary butyl alcohol was reacted with 25.0 ml of 2,4-dinitrophenylhydrazine reagent; 2) 20 ml of tertiary butyl alcohol containing the carbonyl compound were reacted with 100 ml of 2,4-dinitrophenylhydrazine reagent, 120 ml of 2*N* HCl, and 250 ml of water, the latter conditions being those used for the Girard T hydrazone conversion and necessary to compare with reaction of the Girard T reagent. The method of reaction in diluted 2*N* HCl is a modified Iddles *et al.* (1939) procedure and is similar to that described in earlier publications.

The 2,4-dinitrophenylhydrazone derivatives were extracted with carbon tetrachloride and benzene (Gaddis *et al.*, 1959), and monocarbonyl derivatives were purified by passage through hydrated alumina as described in previous publications (Gaddis *et al.*, 1959, 1960; Gaddis and Ellis, 1959).

When determinations were made on fat, the grade of alumina and solvent systems described by Schwartz and Parks (1961) and Keeney *et al.* (1962) were used to obtain clean separation of the simple monocarbonyl derivatives from the ketoglyceride derivatives. Appropriate blanks were run for all steps and checked for artifacts.

The amount of aldehyde or ketone was determined by obtaining absorption data and calculating micromolar amounts from average extinction coefficients. All absorbance values were based on the amount in 100.0 ml of carbon tetrachloride solution, and absorbance values used for 1 micromole were 0.205/343-52 $m\mu$ for 2-alkanones and alkanals, 0.276/355-65 $m\mu$ for alk-2-enals, and 0.373/370-380 for alk-2,4-dienals (Ellis and Gaddis, 1959).

RESULTS AND DISCUSSION

Reaction of Girard T reagent with carbonyl compounds. The completeness of reaction of the Girard T reagent with carbonyl compounds was evaluated by conversion of the hydrazone derivatives to 2,4-dinitrophenylhydrazones. Condensation of n-aliphatic aldehydes and 2-alkanones with 2,4-dinitrophenylhydrazine is generally considered to be quantitative. However, Begemann and De Jong (1959) studied methods of reaction reported in the literature for small amounts of aldehydes and ketones and found much variation in completeness of reaction. Those investigators showed that passing a solution of carbonyl compound in petroleum ether through a Celite column charged with 2N HCl and 2,4-dinitrophenylhydrazine gave quantitative results consistently. Direct reaction of aldehydes and 2-alkanones with 2,4-dinitrophenylhydrazine by procedure 1 gave quantitative results. This method was used to check the contents of monocarbonyl compounds in stock solutions. Table 1 shows the completeness of condensation of n-aliphatic aldehydes with 2,4-dinitrophenylhydrazine by procedure 2, the method

employed in this work. These data indicate that reaction under these conditions was virtually quantitative. However, as shown in Table 2, the formation of 2-alkanone derivatives by similar reaction conditions was very much less satisfactory. With the exception of acetone, 2-alkanones with carbon chains up to about C-8 reacted poorly. The great dilution necessary in the Girard T procedure was apparently responsible for this (Cheronis *et al.*, 1957). In connection with this result, Stone *et al.* (1962) reported that 2-heptanone by the gravimetric method of Iddles *et al.* (1939) reacted only to the extent of 63%. Addition of 50 ml of carbon tetrachloride to the reaction mixture brought all yields to a quantitative level. The reason for the effectiveness of the carbon tetrachloride extraction during the reaction may be the breaking of an equilibrium by removal of hydrazones as formed. The higher-molecular-weight 2-alkanones form hydrazones that are more insoluble in the reaction mixture, and the condensation goes to completion. The solvent extraction did not affect recoveries of n-aldehydes and the higher alkanones, and was used regularly in the remaining experiments.

The reaction of n-aliphatic aldehydes with the Girard T reagent at room temperature under anhydrous conditions is shown in Table 3. The data indicate virtually quantitative reaction under neutral conditions or in the presence of acetic acid, except for very high-molecular-weight aldehydes. The amount of carbonyl compound present was determined by direct reaction with 2,4-dinitrophenylhydrazine; therefore, it follows that the conversion of Girard T hydrazone to 2,4-dinitrophenylhydrazone was very effective. The reaction appeared not to be influenced by the presence of acetic acid. Acetic acid was originally recommended for the Girard T reaction (Girard and Sandulesco, 1963) and definitely aids the condensation with some 2-alkanones.

The completeness of reaction of 2-alkanones at room temperature under neutral and anhydrous conditions was related to the molecular weight. As shown in Table 4, acetone reacted quantitatively; but, with increase in carbon chain, there was a steady decline in completeness of combination. The

Table 1. Reaction of aldehydes with 2,4-dinitrophenylhydrazine.

n-Aldehydes	Weight (mg)	Absorbance		
		Theoretical	Found	% Recovery
Alkanals				
C-4	1.088	3.075	3.145	102
	0.242	0.688	0.658	96
C-5	0.242	0.576	0.547	95
C-7	1.758	3.16	3.19	101
	0.244	0.439	0.410	93
	0.049	0.088	0.080	91
C-9	0.248	0.357	0.322	93
C-10	0.468	0.614	0.618	101
C-12	0.25	0.278	0.252	91
	0.05	0.056	0.061	109
Alk-2-enal				
C-4	0.571	2.25	2.268	101
	0.258	1.014	0.997	98
C-7	0.963	2.37	2.32	98
C-10	1.094	1.96	1.84	94
Alk-2,4-dienal				
C-6	0.596	2.314	2.372	106
C-9	0.282	0.76	0.768	101
C-10	1.040	2.55	2.43	95
C-15	1.305	2.195	2.06	94

Table 2. Reaction of 2-alkanones with 2,4-dinitrophenylhydrazine.

2-Alkanone	Weight (mg)	Absorbance				
		Theoretical	Found	%	Carbon tetrachloride ^b	
					Found	%
C-3	1.022	3.612	3.400	95	3.72	103
	0.235	0.830	0.788	94
C-4	1.110	3.163	1.92	61	3.40	107
C-5	1.412	3.366	1.58	47	3.42	102
	0.241	0.573	0.622	109
C-7	1.874	3.370	2.15	64	3.32	99
	0.285	0.515	0.546	106
C-9	2.36	3.407	3.470	102
C-11	2.62	3.161	3.370	107
C-13	0.248	0.250	0.269	106
	3.390	3.506	3.650	104
C-17	3.950	3.188	3.220	101
C-18	4.395	3.19	3.25	102

* Carbon tetrachloride in reaction mixture.

prescribed use of acetic acid increased yields to a quantitative level up to about C-11, but showed small benefit for the higher-molecular-weight ketones. This appears not to

be due to an equilibrium state, since the high-molecular-weight aldehydes also reacted incompletely.

Recovery of carbonyl compounds from

Table 3. Reaction of n-aldehydes with Girard T reagent under anhydrous conditions.

Aldehydes	Present	Absorbance			
		Neutral		Acetic acid	
		Found	% recovered	Found	% recovered
n-Alkanals	0.118	0.114	97
C-3	0.123	0.115	94	0.132	107
	0.643	0.621	97	0.641	100
	0.780	0.800	103
	0.622	0.636	102	0.621	100
C-4	0.547	0.534	98	0.606	111
C-5	2.040	1.970	97	1.870	91
	0.084	0.080	95
C-7	0.388	0.374	96	0.418	108
	2.570	2.500	99
	0.308	0.319	104	0.349	113
C-9	2.320	2.244	97	2.322	100
C-10	0.058	0.049	85
C-12	0.262	0.266	102	0.283	108
	2.45	1.010	41	1.335	54
C-16	2.45	1.010	41	1.335	54
Alk-2-enal					
C-4	0.196	0.203	104
	0.997	1.022	103	1.03	103
C-9	5.442	5.03	92
C-10	2.212	2.01	91	2.215	100
	2.212	2.09	95
Alk-2,4-dienal:					
C-9	0.768	0.730	95	0.750	100
C-10	2.015	1.987	99
	2.158	1.87	87
C-15	2.158	1.405	65	1.055	49

Table 5. Recovery from fat under anhydrous conditions with Girard T reagent.

Monocarboxyl compounds	Absorbance				
	Present	Neutral		Acetic acid	
		Found	% recovered	Found	% recovered
Alkanal					
C-3	1.741	1.639	94
C-5	1.391	1.382	99
	1.786	1.724	97
	2.600	2.315	89
	2.170	1.995	92
C-7	1.861	1.699	91
	1.978	1.814	92
C-10	3.054	2.034	67
C-12	2.090	2.035	97
	2.158	1.53	71
C-16	2.450	0.74	30	1.615	66
Alk-2-enal					
C-4	3.950	3.244	82
	2.272	1.970	87
	2.272	1.845	81
C-7	2.303	1.008	44
	2.372	2.14	90
C-10	2.212	2.34	106
	1.878	1.000	53
Alk-2,4-dienal					
C-6	2.328	1.928	83
	2.180	2.00	92
C-10	2.403	0.776	32
	2.140	1.96	92
	2.238	1.96	88
C-15	2.158	0.22	10	0.82	38
2-Alkanone					
C-4	2.061	1.990	97
C-7	2.138	0.350	16	1.575	74
	2.138	0.240	11	1.465	69
C-11	2.658	0.170	6	1.015	38
C-13	2.548	0.035	1	0.541	21
C-17	2.318	0.00	0	0.145	6

pH 3.0, but a slowing of reaction rate took place above pH 5.0. This was not investigated further, since satisfactory reaction of aldehydes was obtained under unbuffered conditions.

Reaction of 2-alkanones with Girard T under aqueous conditions in the presence of fat was poor. As shown in Table 7, yields decreased with length of carbon chain, and acetic acid again depressed the degree of reaction. Tests with pH 3.0-7.0 buffers and 0.1-0.2 mole sodium acetate with acetic acid showed some small improvement in recovery at the higher pH range. Reaction had reached equilibrium in less than 2 hr,

and extension of reaction times to as long as 16 hr did not improve yields significantly.

Recovery of 2-alkanones. Achievement of quantitative or even uniform recovery of 2-alkanones from fat is elusive. However, these compounds from C-3 to C-11 reacted quantitatively with Girard T reagent in the presence of acetic acid under anhydrous conditions without the fat (Table 4). Investigators (Wheeler, 1962) have used ethanol or methanol for other applications with a degree of success. The effect of solvents when the reaction was run in the presence of fat under anhydrous conditions is shown in Table 8. Ethanol and methanol improved

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Table 6. Recovery of aldehydes from fat under aqueous conditions with Girard T reagent.

n-Aldehydes	Absorbance				
	Present	Neutral		Acetic acid	
		Found	% recovered	Found	% recovered
Alkanal					
C-3	1.741	1.756	101
C-5	1.391	1.391	100
	2.600	2.230	86
C-7	1.861	1.843	99
C-10	3.054	2.968	97
C-12	2.09	1.65	79
C-16	1.90	1.62	85
	2.45	2.11	86	0.72	29
Alk-2-enal					
C-4	2.072	1.995	96
	2.272	0.83	37
C-7	1.772	1.725	97
	2.372	2.010	85
C-10	1.842	1.790	97
	2.122	1.730	78
Alk-2,4-dienal					
C-6	2.31	2.17	95
	2.18	2.165	99
C-10	2.06	2.07	101
	2.238	2.084	93
C-15	2.060	1.92	93
	2.158	0.605	28

reaction considerably, though not to a quantitative level. Ethanol or methanol might be useful in obtaining a more representative isolation of lower-molecular-weight 2-alkanones. Acetic acid or a longer reaction time of 16 hr did not increase the yield very appreciably except in the case of tertiary butyl alcohol and isopropyl alcohol. Isopropyl alcohol, used recently by Stanley *et al.* (1961), favored 2-alkanone isolation somewhat better than tertiary butyl alcohol.

Effects of solvent, temperature and acid.
A comparison of the use of the four differ-

ent solvents for fat under anhydrous conditions shown in Table 9 indicates a lack of uniformity in reaction with representatives of the three aldehyde classes. The primary alcohols accelerated reaction but were incomplete with alkanal C-12 after 16 hr of reaction. Tertiary butyl alcohol determinations were incomplete with alkanal C-12 and alk-2-enal C-10, but quantitative with alk-2,4-dienal C-10 at the longer reaction time.

Girard and Sandulesco (1936) originally recommended refluxing, and this step has

Table 7. Reaction of 2-alkanones with Girard T under aqueous conditions in the presence of fat.

2-Alkanones	Absorbance				
	Present	Neutral		Acetic acid	
		Found	% recovered	Found	% recovered
C-4	2.061	2.105	102
	2.228	1.86	84
C-7	2.138	1.100	51	0.99	46
C-11	2.658	1.27	48	0.67	25
C-13	2.548	0.44	17	0.35	14
C-17	2.318	0.30	13	0.04	2

Table 8. The effect of anhydrous solvent on reaction of C-11 2-alkanones in the presence of fat with Girard T reagent.

				Absorbance		
Solvent (20 ml)	Present	Neutral			Acetic acid	
		Found	% recovered		Found	% recovered
3-hr reaction						
Tertiary BuOH	2.658	0.13	5	2.658	0.575	22
Isopropyl alcohol	2.658	0.20	8	2.578	0.81	32
Ethyl alcohol	2.578	1.47	57	2.578	1.75	69
Methyl alcohol	2.678	1.18	44	2.678	1.435	54
16-hr reaction						
Tertiary BuOH	2.658	0.145	6			
Isopropyl alcohol	2.578	0.746	29			
Ethyl alcohol	2.578	1.37	53			
Methyl alcohol	2.678	1.37	51			

been more or less adhered to through the intervening years. This work indicated that, for the compounds examined, refluxing was either unnecessary, ineffective, or detrimental. Heating actually tended to reduce recovery, and the effect was particularly sharp in the case of alk-2-enals and alk-2,4-dienals. An adverse affect of heat in the Girard T reaction has also been commented on by Wheeler *et al.* (1961). Where reaction was reluctant, as in the 2-alkanones, there was no indication that refluxing aided reaction. Where primary and secondary alcohols were used for solvents, refluxing greatly increased the amount of dehydro-

genation (Gaddis *et al.*, 1961) and the carbonyl compound artifacts. For some applications, it is fortunate that the reaction goes without heat, which would break down carbonyl precursors (Gaddis *et al.*, 1960).

Teitelbaum (1958) suggested using a cation-exchange resin, Amberlite IRC 50, as a substitute for glacial acetic acid. This modification has been used recently by several investigators (Gaddis *et al.*, 1960, Stanley *et al.*, 1961). Amberlite IRC 50 and a more acid cation-exchange resin, Dowex 50, were used in numerous comparisons under the conditions described above. They showed nothing of advantage as cata-

Table 9. The effect of anhydrous solvent on the reaction of aldehydes in the presence of fat with Girard T reagent.

Solvent (20 ml)	Absorbance				
	Present	Reaction 3 hr		Reaction 16 hr	
		Found	% Recovery	Found	% Recovery
Alkanal C-12					
Tertiary BuOH	2.158	1.53	71	1.32	61
Isopropyl alc.	2.228	0.60	27	1.135	51
Ethanol	2.228	1.71	77	1.65	74
Methanol	2.198	1.84	84	1.86	85
Enal C-10					
Tertiary BuOH	1.878	1.00	53	1.27	68
Isopropyl alc.	1.618	1.40	86	1.52	94
Ethanol	1.908	1.88	99	2.11	109
Methanol	1.918	1.59	82	1.54	79
2,4-Dienal C-10					
Tertiary BuOH	2.158	1.01	47	2.105	98
Isopropyl alc.	1.178	1.58	73	1.925	88
Ethanol	2.228	1.95	88	2.200	99
Methanol	2.228	1.59	71	2.12	95

lysts for the condensation. Amberlite IRC 50 was completely ineffective as a replacement for glacial acetic acid in the reaction of 2-alkanones with Girard T reagent. These resins, especially the Dowex 50, tended to decrease recoveries, and this was particularly serious in the case of the unsaturated aldehydes. Some of this loss may be due to irreversible holding of the Girard T hydrazone by the resin (Hornstein, 1962). On the other hand, the Dowex 50 resin has shown promise as a useful means of decomposition of hydroperoxides into carbonyl compounds (Gaddis *et al.*, 1960).

Sensitivity and utility of the method. The sensitivity of the determination began to fall off at concentrations lower than 0.5 μ m carbonyl/100 ml. This decline was due to the increasing significance of variation in the blank. The blank was composed of unremovable impurities formed from the carbonyl reagents. These reaction by-products had an absorption maximum in carbon tetrachloride at about 340 m μ .

The method is believed to be useful for the isolation of aldehydes from oxidized fats and oils. Complete evaluation of the reagent's effect on hydroperoxides and other oxidative carbonyl precursors is considered beyond the scope of this paper. It has been established that aldehydes can be isolated quantitatively from fat by this procedure. In view of earlier work by the authors (Gaddis *et al.*, 1960), the mild conditions employed might be expected to recover aldehydes to a degree approaching those that are free. Removal of only the free carbonyl compounds may be an elusive achievement. According to Lea and Swoboda (1962), there appears to be some formation or release of volatile carbonyl compounds from non-volatile precursors even under the mild conditions of vacuum distillation at 50°C. This may explain the isolation of more carbonyl compounds upon repeated treatment with the Girard T reagent in our earlier study (Gaddis *et al.*, 1960). Preliminary studies have shown that this Girard T reagent procedure extracts much less aldehyde than the steam distillation method, and that values approach those obtained by the vacuum distillation technique of Lea and Swoboda (1962). The Girard T reagent under

these mild optimum conditions hydrolyzes aldehyde diethyl acetals. However, it does not split the enol ether-linked aldehydes present in fresh fats (Schogt *et al.*, 1960; Parks *et al.*, 1961). Reaction with the naturally occurring ketoglycerides (Keeney *et al.*, 1962) was incomplete.

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